

# Polycystin-2 Activation by Inositol 1,4,5-Trisphosphate-induced $\text{Ca}^{2+}$ Release Requires Its Direct Association with the Inositol 1,4,5-Trisphosphate Receptor in a Signaling Microdomain<sup>\*S</sup>

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Autosomal dominant polycystic kidney disease is characterized by the loss-of-function of a signaling complex involving polycystin-1 and polycystin-2 (TRPP2, an ion channel of the TRP superfamily), resulting in a disturbance in intracellular  $\text{Ca}^{2+}$  signaling. Here, we identified the molecular determinants of the interaction between TRPP2 and the inositol 1,4,5-trisphosphate receptor ( $\text{IP}_3\text{R}$ ), an intracellular  $\text{Ca}^{2+}$  channel in the endoplasmic reticulum. Glutathione *S*-transferase pulldown experiments combined with mutational analysis led to the identification of an acidic cluster in the C-terminal cytoplasmic tail of TRPP2 and a cluster of positively charged residues in the N-terminal ligand-binding domain of the  $\text{IP}_3\text{R}$  as directly responsible for the interaction. To investigate the functional relevance of TRPP2 in the endoplasmic reticulum, we re-introduced the protein in TRPP2<sup>-/-</sup> mouse renal epithelial cells using an adenoviral expression system. The presence of TRPP2 resulted in an increased agonist-induced intracellular  $\text{Ca}^{2+}$  release in intact cells and  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release in permeabilized cells. Using pathological mutants of TRPP2, R740X and D509V, and competing peptides, we demonstrated that TRPP2 amplified the  $\text{Ca}^{2+}$  signal by a local  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release mechanism, which only occurred in the presence of the TRPP2- $\text{IP}_3\text{R}$  interaction, and not via altered  $\text{IP}_3\text{R}$  channel activity. Moreover, our results indicate that this interaction was instrumental in the formation of  $\text{Ca}^{2+}$  microdomains necessary for initiating  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. The data strongly suggest that defects in this mechanism may account for the altered  $\text{Ca}^{2+}$  signaling associated with pathological TRPP2 mutations and therefore contribute to the development of autosomal dominant polycystic kidney disease.

Autosomal dominant polycystic kidney disease (ADPKD)<sup>4</sup> is an inherited human disorder that affects more than six million people worldwide and is the most common monogenic cause of kidney failure in humans (1). ADPKD results in end-stage renal disease in ~50% of the affected individuals by the age of 60. ADPKD arises as a consequence of mutations of two genes *PKD1* and *PKD2*, encoding integral membrane proteins polycystin-1 (PKD1, ~460 kDa) and polycystin-2 (TRPP2, ~110 kDa), respectively. Most mutations identified in affected families appear to truncate and (or) inactivate either of both proteins (2–5). Mutations in *PKD1* account for the vast majority (~85%) of patients with ADPKD and are associated with a more severe clinical presentation and earlier onset of end-stage renal disease than the *PKD2* phenotype (4). However, in all other aspects, *PKD1* and *PKD2* mutations produce virtually indistinguishable disease manifestations, indicating that the two proteins might function in a common signaling pathway involved in maintaining the terminally differentiated state of renal epithelial cells.

TRPP2 is a 968-amino acid (aa) protein with six predicted transmembrane domains and is highly conserved among multicellular organisms and widely expressed in various tissues (2). Structural analyses indicate that TRPP2 contains several functional domains in its C-terminal tail. There are two  $\text{Ca}^{2+}$ -binding sites (aa 680–796) arranged in a typical and an atypical EF-hand motif, which could be involved in a  $\text{Ca}^{2+}$ -mediated regulation of TRPP2 (6). An endoplasmic reticulum (ER) retention signal (aa 787–820) (7) and a coiled-coil domain (aa 839–919), responsible for homo- and heterodimerization (8, 9), are also present. Recently, it was reported that this coiled-coil domain was responsible for formation of a TRPP2 trimer that interacts with PKD1 in the plasma membrane (9).

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<sup>4</sup> The abbreviations used are: ADPKD, autosomal dominant polycystic kidney disease; AC, acidic cluster;  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release;  $[\text{Ca}^{2+}]_{\text{ER}}$ ,  $\text{Ca}^{2+}$  concentration in the ER;  $[\text{Ca}^{2+}]_{\text{cyt}}$ , cytosolic  $\text{Ca}^{2+}$  concentration; ER, endoplasmic reticulum;  $\text{IP}_3\text{R}$ , inositol 1,4,5-trisphosphate receptor;  $\text{IICR}$ , inositol 1,4,5-trisphosphate-induced  $\text{Ca}^{2+}$  release; LBD, ligand-binding domain; PKD1, polycystin-1; RyR, ryanodine receptor; TG, thapsigargin; TRPP2, polycystin-2; aa, amino acid(s); MOPS, 4-morpholinepropanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; GST, glutathione *S*-transferase; TBS, Tris-buffered saline; CT, C-terminal; NT, N-terminal; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N'*,*N'*-tetraacetic acid.

There is a long-standing debate on the subcellular localization of TRPP2. TRPP2 has been detected (i) in the plasma membrane, where it is supposed to form a receptor-operated, non-selective cation channel (10), (ii) in the primary cilium, where it could act as a mechanosensitive channel, possibly in association with PKD1 (11), TRPC1 (12, 13), or TRPV4 (11, 14), (iii) in the ER, where it is proposed to function as an intracellular  $\text{Ca}^{2+}$ -release channel (15), but also in centrosomes and mitotic spindles of dividing cells (reviewed in Refs. 16–18). The trafficking of TRPP2 to these specific subcellular compartments is regulated by (i) specific motifs within the protein (7, 19), (ii) multiple protein-protein interactions, *e.g.* with phosphofurin acidic cluster sorting proteins 1 and 2 (20), and (iii) a casein kinase 2-mediated phosphorylation of Ser<sup>812</sup> in the C terminus of TRPP2 (20, 21). However, the predominant subcellular localization of TRPP2 is in the ER, as shown by sensitivity to Endo H, immunofluorescence, co-localization, and co-distribution with ER-resident proteins (7, 15).

Interaction between TRPP2 and the two major intracellular  $\text{Ca}^{2+}$ -release channels, the ryanodine receptor (RyR), and the inositol 1,4,5-trisphosphate receptor ( $\text{IP}_3\text{R}$ ), has been reported. Biochemical and functional assays have demonstrated that the N-terminal part of TRPP2 is sufficient to bind the cardiac RyR2, whereas the C-terminal part of TRPP2 can only bind to RyR2 when it is in the open state, thereby inhibiting RyR function (22). Co-immunoprecipitation assays indicated that TRPP2 physically interacts with the  $\text{IP}_3\text{R}1$ , most likely through its C-terminal part (23). Recently Li *et al.* (24) demonstrated also that ER-localized PKD1 can interact with the  $\text{IP}_3\text{R}$ , thereby inhibiting  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release (IICR). Several studies observed an enhancement of intracellular  $\text{Ca}^{2+}$  release that was attributed to an effect of TRPP2 (15, 23, 25, 26), but the physiological mechanism of action was not elucidated. On the other hand, Wegierski and co-workers (27) observed that TRPP2 could also act as a passive  $\text{Ca}^{2+}$ -leak channel in the ER, thereby lowering the  $\text{Ca}^{2+}$  concentration in the ER ( $[\text{Ca}^{2+}]_{\text{ER}}$ ), which resulted in an opposite effect and decreased the magnitude of IICR. These controversial results illustrate that the exact mechanism through which polycystins in general and TRPP2 in particular modulate  $\text{Ca}^{2+}$  signaling is not yet understood. We therefore performed a detailed analysis of the molecular and functional relationship between the  $\text{IP}_3\text{R}$  and TRPP2.

Here, we identified a conserved positively charged cluster in the N-terminal suppressor domain of the  $\text{IP}_3\text{R}$  and an acidic cluster located at the end of the ER-retention signal in the C-terminal tail of TRPP2 as being crucial for the interaction between both proteins. Moreover, in a background of renal epithelial TRPP2<sup>-/-</sup> cells, we observed a clear potentiation of both ATP-induced intracellular  $\text{Ca}^{2+}$  release in intact cells and IICR in permeabilized cells upon TRPP2 re-introduction. Further analysis using pathological mutants of TRPP2 and peptides that compete with the interaction between TRPP2 and the  $\text{IP}_3\text{R}$  revealed that the observed increase in IICR required both a functional TRPP2 channel and a physical interaction with the  $\text{IP}_3\text{R}$ . We suggest that TRPP2 functions as a tightly regulated channel in the ER that participates in an intracellular signaling complex together with the  $\text{IP}_3\text{R}$ , thereby stimulating intracel-

lular  $\text{Ca}^{2+}$  release within a microdomain in the immediate neighborhood of these interacting proteins.

## EXPERIMENTAL PROCEDURES

**Materials**—Isopropyl  $\beta$ -D-thiogalactoside, bovine serum albumin, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone,  $\text{IP}_3$ , and ionomycin were from Sigma. Fura2-AM was from TEF Labs (Austin, TX). MagFluo4-AM, nickel-nitrilotriacetic acid-agarose resin, NuPAGE® gels, NuPAGE loading dye solution sample buffer, MOPS buffer, the Virapower kit, geneticin, all cell culture media, and supplements were from Invitrogen. ATP was from Roche Applied Science. Thapsigargin (TG) was from Alexis (Zandhoven, Belgium). Glutathione-Sepharose 4B, secondary antibodies coupled to horseradish peroxidase, Enhanced Chemiluminescence (ECL) reagents, and Hyperfilms were from GE Healthcare. r-Protein A-TSK resin was from Affilind (Liège, Belgium). Immobilion-P polyvinylidene fluoride microporous transfer membranes were from Millipore (Billerica, MA). 96-Well culture plates (CELLSTAR, black polystyrene,  $\mu$ Clear flat bottom with lid) were from Greiner (Wemmel, Belgium). The QuikChange Site-directed Mutagenesis kit was from Stratagene (La Jolla, CA). Slide-A-Lyzer dialysis cassettes and peptides were from Thermo Fisher Scientific (Ulm, Germany). Peptides were synthesized with a purity of >70% and verified by mass spectrometry and high pressure liquid chromatography.

**DNA Constructs and Adenovirus Construction**—The original cDNA of mouse wild-type TRPP2, which was subcloned in a pcNeo/IRES-GFP vector with a C-terminal myc tag, was a kind gift from Dr. V. Gerke, University of Münster, Germany. Using this construct we generated an LLC-PK1 cell line (porcine renal epithelial cells) stably expressing wild-type TRPP2 by geneticin selection and subsequent fluorescence-activated cell sorter selection of a green fluorescent protein-expressing cell population.

An adenovirus was produced using the Virapower kit from Invitrogen for wild-type TRPP2 and two pathological mutants D509V and R740X. A control virus was made starting from the empty pAd/PL-DEST vector. Using Gateway technology first a pDONR221-TRPP2 wild-type construct under control of a cytomegalovirus promoter was made via a BP recombination reaction (between *attB*- and *attP*-containing substrates). Subsequently an LR recombination reaction (between *attL*- and *attR*-containing substrates) was performed to obtain destination vector pAd/PL-DEST-TRPP2. These were then transfected using Lipofectamine in HEK 293A cells, which were genetically modified and include human adenovirus type-5 DNA, to facilitate production of adenovirus. The TRPP2 D509V and R740X mutants were made by site-directed mutagenesis by introducing GTT (V) to replace GAT (D) at codon 509, and TAG (Stop) to replace CGG (R) at codon 740, respectively, with wild-type pDONR221-TRPP2 as donor DNA. The different domains of the  $\text{IP}_3\text{R}1$  (domains 1 to 6), the complete ligand-binding domain (LBD, aa 1–604), and the suppressor domains (aa 1–225) of  $\text{IP}_3\text{R}1$  and  $\text{IP}_3\text{R}3$  were subcloned in the bacterial pGEX-6p2 vector with an N-terminal glutathione S-transferase (GST) tag, as previously described (28, 29).



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The cDNA fragments corresponding to the complete cytosolic N terminus (TRPP2-NT, aa 1–221) and C terminus (TRPP2-CT, aa 679–966) of TRPP2 were subcloned into the bacterial pET21b and pGEX-6p2 vector, to obtain HIS- or GST-tagged proteins, respectively. All constructs were sequenced on a Genetic Analyzer 3100 using Big Dye Terminator V1.1 technology (Applied Biosystems, Foster City, CA).

**Cell Culture**—TRPP2<sup>−/−</sup> (2D2) renal proximal tubulus epithelial cells were derived from a TRPP2<sup>−/WS25</sup> mouse, transgenic for the SV40 large T-antigen (30). The cells were isolated and cultured as previously described (31). Cells were transduced by adding the adenovirus to the normal cell culture medium. For transduction of the cells, viral titers were determined for wild-type TRPP2 (1/1000), TRPP2 D509V (1/704), and TRPP2 R740X (1/1124) to obtain an equal amount of protein expression, as assayed by Western blotting. We chose in all functional experiments a moderate overexpression using the adenoviral system, mimicking endogenous levels in wild-type kidney cells instead of a massive overexpression of the protein, to avoid induction of ER stress responses.

**Antibodies**—For wild-type TRPP2, a mouse monoclonal antibody (sc-28331) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) directed against its C-terminal tail (aa 689–968) was used at a dilution of 1/1000 in Western blotting analysis. Additionally, a rabbit polyclonal antibody directed against the N terminus (aa 103–203) (YCB9) was a kind gift from Dr. Cai and Dr. Somlo and was used to recognize wild-type TRPP2 and the D509V and R740X mutants (7). This antibody was used at a 1/5000 dilution for Western blotting analysis and 1/500 dilution for immunofluorescence. The mouse monoclonal antibody (610312) that specifically recognizes IP<sub>3</sub>R3 (aa 22–230) was from BD Biosciences (Pharmingen), and used at a 1/1000 dilution in Western blotting. Immunological detection of IP<sub>3</sub>R1 was carried out using a Rbt03 polyclonal antibody raised against C-terminal amino acids 2735–2749 of mouse IP<sub>3</sub>R1 (32), at a 1/3000 dilution in Western blotting. For immunological detection of the recombinant HIS fusion proteins, a horseradish peroxidase-conjugated Penta-HIS antibody was used from Qiagen (Venlo, The Netherlands).

**Immunoprecipitation**—LLC-PK1 cells stably expressing wild-type TRPP2 were lysed in lysis buffer (50 mM Tris, 0.3 M NaCl, 1% Triton, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 5 μM leupeptin, pH 7.5). The lysate (200 μg/sample) was cleared by centrifugation (5 min, 10,000 × g, 4 °C). Supernatants were incubated for 90 min at 4 °C while gently mixing (1400 rpm) with antibodies (~1.5 μg): (i) anti-TRPP2 (Santa Cruz Biotechnology Inc.), (ii) anti-IP<sub>3</sub>R1, (iii) anti-IP<sub>3</sub>R3, (iv) nonspecific mouse IgG (Santa Cruz Biotechnology Inc., sc-2025), or (v) nonspecific rabbit IgG (Santa Cruz Biotechnology Inc., sc-2027). Subsequently, r-protein A-TSK-Sepharose resin (50% in lysis buffer) was added and incubation was continued for another hour. Beads were collected by centrifugation (20 s, 2000 × g) and washed once with Tris-buffered saline (TBS) supplemented with 0.25% Triton X-100 and 0.1 M LiCl and three times with TBS supplemented with 0.25% Triton X-100. Finally, the beads were resuspended in 30 μl of NuPAGE loading dye solution sample buffer and

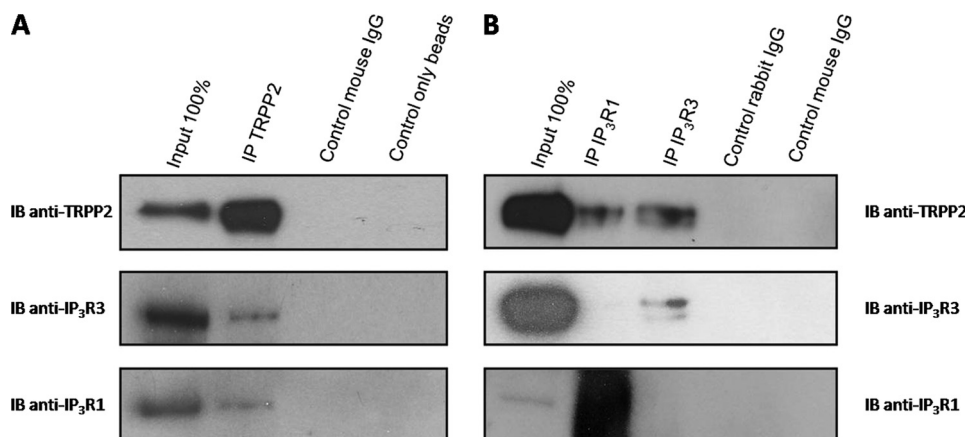
heated for 10 min at 75 °C. Supernatants were further analyzed by SDS-PAGE and Western blotting.

**Preparation of GST or HIS Fusion Proteins**—pGEX-6p2 and pET21b constructs were transformed into BL21 *Escherichia coli*. Colonies were grown overnight in 50 ml of dYT medium (16 g/liter of peptone, 10 g/liter of yeast extract, 5 g/liter of NaCl, pH 7.4) at 37 °C. dYT medium (~400 ml) was added to this preculture, and bacteria were further grown at 28 °C until  $A_{600}$  reached 0.8–1. Protein expression was induced by adding 0.1 mM isopropyl β-D-thiogalactoside and bacteria were further grown at 28 °C for 4–8 h or at 14 °C for 20 h. Bacterial cells were harvested and lysed by sonication (9 × 10 s, 12 kHz). Lysates were cleared by centrifugation (30 min, 15,000 × g). The soluble fractions were then incubated during 2 h with glutathione-Sepharose 4B or nickel-nitrilotriacetic acid-agarose beads at 4 °C. After washing the beads, fusion proteins were eluted with 10 mM glutathione or 250 mM imidazole, respectively. Purified proteins were dialyzed overnight against TBS, using Slide-A-Lyzer dialysis cassettes with a cut-off of 10 kDa.

**GST Pulldown**—Purified and dialyzed GST fusion proteins or parental GST (control) were incubated with purified and dialyzed HIS fusion proteins or a cleared lysate from LLC-PK1 cells expressing TRPP2 in pulldown buffer (50 mM Tris, 1 mM EGTA, pH 7.4) and immobilized on glutathione-Sepharose 4B beads via rotation in a head-over-head rotator for 1–2 h at 4 °C. The beads were washed 4 times with pulldown buffer and complexed GST fusion proteins were eluted in 100 mM glutathione in pulldown buffer. Eluates were further analyzed using SDS-PAGE and Western blotting.

**SDS-PAGE and Western Blotting**—Protein samples were analyzed by NuPAGE 4–12% BisTris SDS-polyacrylamide gels using MOPS buffer. After semi-dry electroblotting onto a polyvinylidene fluoride membrane, blocking with TBS containing 0.1% Tween and 5% nonfat dry milk powder or bovine serum albumin, and incubation with primary antibody (in TBS supplemented with 0.1% Tween and 1% nonfat dry milk powder), the blots were incubated with the horseradish peroxidase-conjugated secondary antibodies (in TBS supplemented with 0.1% Tween and 1% nonfat dry milk powder). The immunoreactive bands were visualized with ECL substrate and exposed to Hyperfilm. The Hyperfilm was developed using a Kodak X-Omat 1000 (Kodak). Total protein content was visualized by Ponceau red staining of the blot after film development. Quantification was done with ImageJ software (rsbweb.nih.gov/ij/).

**[Ca<sup>2+</sup>] Measurements**—For measurements of the free cytosolic [Ca<sup>2+</sup>] ([Ca<sup>2+</sup>]<sub>cyt</sub>) in intact cells, the cells were loaded with Fura2-AM (1.25 μM) in modified Krebs buffer (130 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 10 mM glucose, 10 mM Hepes, pH 7.4) for 45 min at room temperature. This solution was then replaced by a modified Krebs buffer without Ca<sup>2+</sup> indicator and the incubation continued for 30–45 min, to allow de-esterification of the loaded dye. 3 mM EGTA was added before each measurement to buffer all free extracellular Ca<sup>2+</sup>. Subsequently, agonist (ATP) or 1 μM ionomycin were added to induce Ca<sup>2+</sup> release from intracellular stores. Fluorescence emission was measured at 510 nm with excitation at 340/380 nm. [Ca<sup>2+</sup>]<sub>cyt</sub> was derived after *in situ* calibration according to the following equation: [Ca<sup>2+</sup>]<sub>cyt</sub> (nM) =  $K_d \times Q \times (R - R_{\min}) /$



**FIGURE 1. Co-immunoprecipitation of full-length TRPP2 and IP<sub>3</sub>R.** *A*, co-immunoprecipitation (IP) of endogenous IP<sub>3</sub>R3 and IP<sub>3</sub>R1 with TRPP2 in TRPP2-expressing LLC-PK1 cells. The upper immunoblot (IB) shows the staining for TRPP2, whereas the lower immunoblots show the staining for IP<sub>3</sub>R3 and IP<sub>3</sub>R1. *B*, co-immunoprecipitation of TRPP2 with endogenous IP<sub>3</sub>R1 or IP<sub>3</sub>R3. The upper immunoblot shows the staining for TRPP2, the middle immunoblot shows the staining for IP<sub>3</sub>R3 and the lower immunoblot shows the staining for IP<sub>3</sub>R1. Representative immunoblots from three independent experiments are shown.

( $R_{\max} - R$ );  $K_d$  is the dissociation constant of Fura2 for  $\text{Ca}^{2+}$  at room temperature (220 nM),  $Q$  is the fluorescence ratio of the emission intensity excited by 380 nm in the absence of  $\text{Ca}^{2+}$  to that during the presence of saturating  $\text{Ca}^{2+}$ ,  $R$  is the fluorescence ratio,  $R_{\min}$  and  $R_{\max}$  are the minimal and maximal fluorescence ratios, respectively.  $R_{\min}$  was measured by perfusion with 10 mM EGTA in  $\text{Ca}^{2+}$ -free Krebs solution and  $R_{\max}$  was obtained by perfusion with 5  $\mu\text{M}$  ionomycin and 5 mM  $\text{CaCl}_2$ . By integrating the traces in Origin 7 (OriginLab Corporation, Northampton, MA), the area under the curve was determined and expressed in arbitrary units.

To measure the free  $[\text{Ca}^{2+}]_{\text{ER}}$ , the cells were loaded with 20  $\mu\text{M}$  MagFluo4-AM in Hepes-buffered saline (135 mM NaCl, 5.9 mM KCl, 11.6 mM Hepes, 1.5 mM  $\text{CaCl}_2$ , 11.5 mM glucose, 1.2 mM  $\text{MgCl}_2$ , pH 7.4) supplemented with 1 mg/ml of bovine serum albumin and 0.2 mg/ml of Pluronic F127 (Invitrogen) as described in Ref. 33. After 60 min, cells were permeabilized in  $\text{Ca}^{2+}$ -free cytosol-like medium containing 20  $\mu\text{g}/\text{ml}$  of saponin (10 min at room temperature).  $\text{Ca}^{2+}$ -free cytosol-like medium had the following composition: 140 mM KCl, 20 mM NaCl, 1 mM EGTA, 2 mM  $\text{MgCl}_2$ , 20 mM Pipes, pH 7.0. Subsequently the solution was replaced with cytosol-like medium without  $\text{Mg}^{2+}$ , but supplemented with carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (10  $\mu\text{M}$ ) and 375  $\mu\text{M}$   $\text{CaCl}_2$  to give a free  $[\text{Ca}^{2+}]$  of 220 nM. 1.5 mM Mg-ATP was added at the beginning of each measurement to load the ER with  $\text{Ca}^{2+}$  via the sarcoplasmic/endoplasmic-reticulum  $\text{Ca}^{2+}$ -ATPase pumps. Upon reaching a maximal loading, IP<sub>3</sub> or ionomycin were added to the cells together with 10  $\mu\text{M}$  TG to prevent re-uptake of  $\text{Ca}^{2+}$  into the ER. Results are calculated as  $F/F_0$  of fluorescence emission at 525 nm after excitation at 490 nm.

Fluorescence was measured using a 96-well microplate reader equipped with automated fluid additions (FlexStation 3, Molecular Devices, Sunnyvale, CA). All experiments were performed at room temperature.

**Confocal Imaging**—Confocal images were made with an LSM510 confocal system on a Zeiss Axiovert 100M microscope (Carl Zeiss Meditec Inc., Jena, Germany) with a 488-nm argon

and 543-nm HeNe laser. The  $\times 40$  plan neofluar, oil-immersion 1.3 NA, DIC objective was used. The images were analyzed with Zeiss LSM Software Release 4.2.

## RESULTS

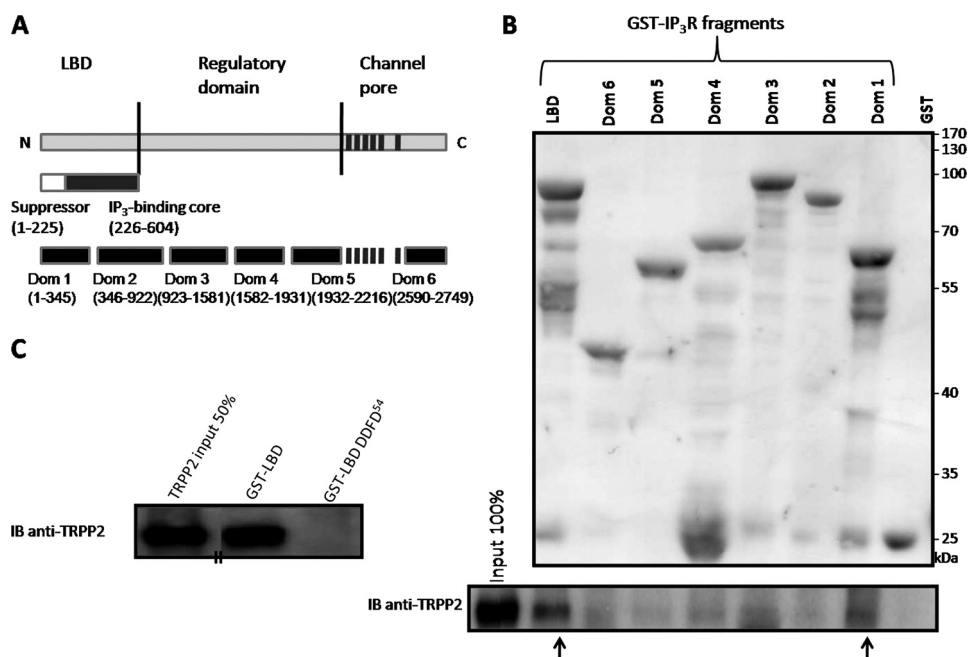
**Full-length TRPP2 Interacts with Endogenous Full-length IP<sub>3</sub>R1 or IP<sub>3</sub>R3**—To investigate whether full-length TRPP2 and the IP<sub>3</sub>R directly interact in intact cells, we performed co-immunoprecipitation experiments (Fig. 1). An LLC-PK1 cell line stably expressing full-length wild-type TRPP2 was used. After cell lysis, immunoprecipitation was performed with an antibody against the C terminus of TRPP2. Specific association between TRPP2 and

endogenous full-length IP<sub>3</sub>R3 or IP<sub>3</sub>R1 was detected by Western blotting analysis (Fig. 1A). The control experiment was performed with nonspecific mouse or rabbit IgG. Furthermore, when an antibody specifically against IP<sub>3</sub>R1 or IP<sub>3</sub>R3 was used for immunoprecipitation, we also identified TRPP2 in the precipitate by Western blotting analysis (Fig. 1B). Due to the insufficient quality of the antibodies available against IP<sub>3</sub>R2, we could not detect or immunoprecipitate IP<sub>3</sub>R2. The data in Fig. 1 indicate that TRPP2 interacts with both IP<sub>3</sub>R1 and IP<sub>3</sub>R3, the latter being the most abundant isoform in LLC-PK1 cells.

**Interaction between TRPP2 and the N-terminal Ligand-binding Domain of the IP<sub>3</sub>R1**—Each of the four IP<sub>3</sub>R monomers can be functionally divided in an N-terminal LBD that binds IP<sub>3</sub>, a central regulatory/coupling domain and a C-terminal channel and gatekeeper domain (reviewed in Ref. 34). The N-terminal LBD (aa 1–604) can be functionally and structurally subdivided into a suppressor domain (aa 1–225) and an IP<sub>3</sub>-binding core (aa 226–604) (35). A linear representation of the IP<sub>3</sub>R is depicted in Fig. 2A. We have employed GST pulldown assays to further characterize the TRPP2-IP<sub>3</sub>R interaction. To map the TRPP2-binding site on the IP<sub>3</sub>R, we used a series of GST-IP<sub>3</sub>R fusion proteins corresponding to five fragments covering the first N-terminal 2216 amino acids and 1 fragment corresponding to the last C-terminal 160 amino acids of mouse IP<sub>3</sub>R1 (28, 29). These fragments are located in the cytoplasm and coincide with “natural” domains generated by limited proteolysis, but the transmembrane domain was not included (36). In addition to these six domains, we used a GST fusion protein covering the complete LBD (aa 1–604). After purification, the recombinant proteins migrated as prominent bands corresponding to their expected molecular weights (Fig. 2B). Lysates obtained from the LLC-PK1 cells stably expressing TRPP2 were incubated with the GST-IP<sub>3</sub>R fragments pre-bound to glutathione-Sepharose 4B resin, with equal loading of the GST-IP<sub>3</sub>R fragments. After pulldown and elution, the samples were processed by SDS-PAGE. TRPP2 was visualized using the mouse monoclonal antibody against TRPP2 (Fig. 2B). Full-length TRPP2 mainly interacted with the complete N-terminal LBD of the



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**FIGURE 2. GST pulldowns using purified IP<sub>3</sub>R subdomains and full-length TRPP2.** A, linear structure of the IP<sub>3</sub>R1: LBD, regulatory domain, and the channel pore. The LBD can be further divided into a suppressor domain (aa 1–225) and an IP<sub>3</sub>-binding core (aa 226–604). GST is N-terminal fused to the LBD and to the six cytoplasmic domains of the IP<sub>3</sub>R1. B, GST pull-down using the different domains of the IP<sub>3</sub>R1, bacterially expressed and purified as GST fusion proteins, and full-length TRPP2, obtained from LLC-PK1 cells stably expressing TRPP2. The upper panel shows Ponceau red staining of the input proteins. The lower immunoblot shows the staining for TRPP2. The arrows indicate the bands with specific interaction. C, pull-down between GST-LBD or GST-LBD mutant (KKFR<sup>54</sup> is mutated to DDFD<sup>54</sup>) and full-length TRPP2, obtained from LLC-PK1 cells stably expressing TRPP2. The immunoblot shows the staining for TRPP2. The short parallel lines indicate the position where a lane was moved from within the same blot.

IP<sub>3</sub>R (aa 1–604) and with domain 1 (aa 1–345), which is part of this LBD (Fig. 2B, lower panel). The interaction was specific, as TRPP2 did not interact with the parental GST.

**A Positively Charged Cluster in the Suppressor Region of the LBD of the IP<sub>3</sub>R Interacts with an Acidic Cluster in the C-terminal Cytoplasmic Tail of TRPP2**—Previous work in our laboratory identified a cluster of positively charged residues (aa 51–54) in the suppressor domain of the IP<sub>3</sub>R1,<sup>5</sup> conserved among all three IP<sub>3</sub>R isoforms, that were critical for interaction with negatively charged protein sequences, as *e.g.* in calmodulin (37). We therefore hypothesized that this positively charged cluster may be involved in the interaction with TRPP2, which contains a highly negatively charged cluster in the C-terminal cytoplasmic tail. We mutated two lysine residues and one arginine residue within this positive cluster to negatively charged aspartate residues (KKFR<sup>54</sup> to DDFD<sup>54</sup>) in the GST-LBD construct (GST-LBD DDFD<sup>54</sup>). Pull-down with GST fusion proteins of the LBD and the LBD DDFD<sup>54</sup> indicated that full-length TRPP2 could interact with GST-LBD but not with GST-LBD DDFD<sup>54</sup> (Fig. 2C).

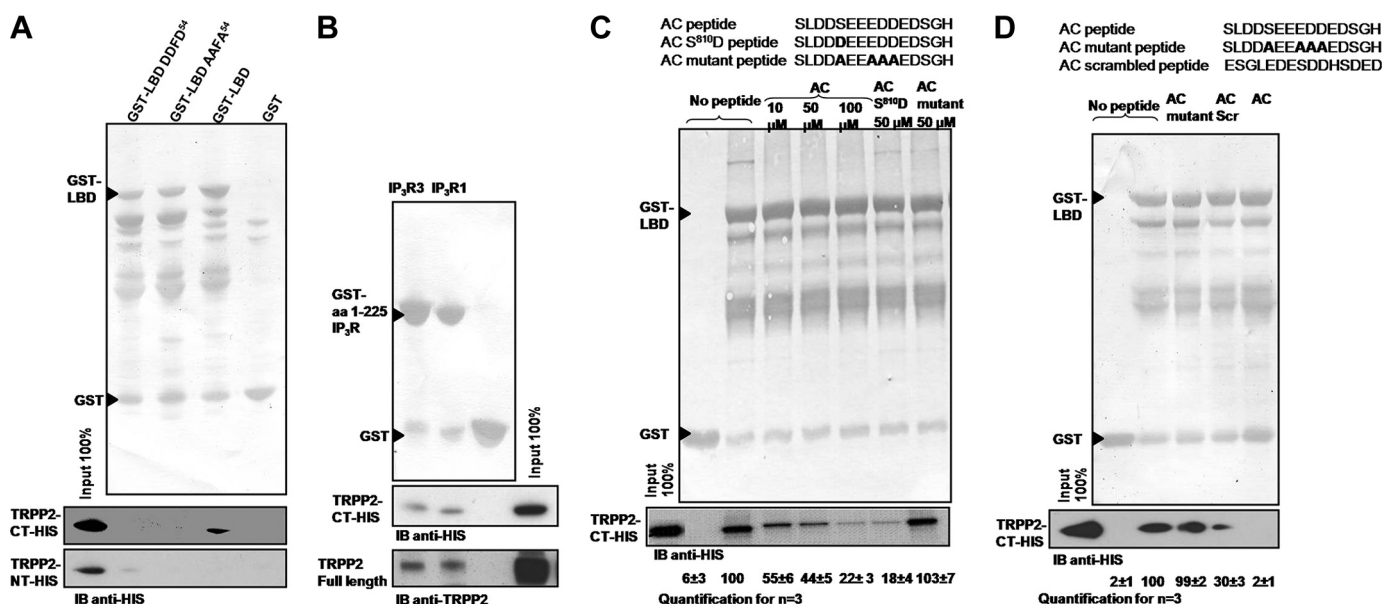
Furthermore, an additional mutant of the LBD was made by mutating the three positively charged residues to neutral residues (KKFR<sup>54</sup> to AAFA<sup>54</sup>) to avoid the too drastic structural changes that can occur by the mutations to negatively charged residues. To identify the region of TRPP2 required for interaction with IP<sub>3</sub>R1, recombinant His<sub>6</sub> fusion proteins of the com-

plete cytosolic N- (aa 1–221) (TRPP2-NT-HIS) and C-terminal part (aa 679–966) (TRPP2-CT-HIS) of TRPP2 were bacterially expressed and purified. Pull-down assays with GST-LBD and both GST-LBD mutants revealed a clear interaction between GST-LBD and TRPP2-CT (Fig. 3A). Like the full-length TRPP2, the interaction with GST-LBD DDFD<sup>54</sup> was abolished. Additionally, interaction with GST-LBD AAFA<sup>54</sup> was similarly abolished. In contrast to TRPP2-CT, TRPP2-NT could neither specifically interact with the LBD nor with its mutants (Fig. 3A), indicating that the C-terminal tail of TRPP2 is the major determinant for binding to the suppressor domain of the IP<sub>3</sub>R. We then tested whether IP<sub>3</sub> could alter the interaction between the IP<sub>3</sub>R and TRPP2, but this was not the case (supplemental Fig. S1). To confirm the interaction with both IP<sub>3</sub>R1 and IP<sub>3</sub>R3, a pull-down was performed between full-length TRPP2 or TRPP2-CT-HIS and the suppressor domains (aa 1–225) of IP<sub>3</sub>R1 and IP<sub>3</sub>R3, fused to GST (Fig. 3B). Both

suppressor domains could pull down TRPP2, both full-length TRPP2 or only its C terminus. TRPP2-CT contains an acidic cluster (aa 810–818 SEEEDEDS) located at the end of the ER retention signal, well conserved among chordates but absent in polycystin-2 like-1 (PKD2L1 or TRPP3) and -2 (PKD2L2 or TRPP5) (supplemental Fig. S2). A casein kinase 2-phosphorylation site (Ser<sup>810</sup> in the mouse sequence, corresponding to Ser<sup>812</sup> in the human sequence) is located within this cluster and has been shown to be important for channel regulation (25). This cluster is also involved in interactions with other proteins (20, 38). To test whether this acidic cluster is implicated in interaction with the IP<sub>3</sub>R, peptides containing this cluster were synthesized. A peptide representing the acidic cluster (SLDD-SEEEDEDSGH) (AC), a phosphomimic mutant (SLD-DDEEEDDSGH) (AC S810D), and a mutant peptide with four alanine mutations replacing the negatively charged amino acids (SLDDAEEAAEDSGH) (AC mutant) were used in GST pull-down assays to compete for the interaction between TRPP2-CT-HIS and GST-LBD. As shown in Fig. 3C, increasing amounts of the AC peptide competed for binding of TRPP2-CT-HIS to GST-LBD. Also the phosphomimic peptide, which contains an extra negative charge, could clearly disrupt the interaction, even more effectively than the wild-type AC peptide (a residual binding of 18 *versus* 44%). The AC mutant peptide, which lacks several negative residues, did not interfere with the binding of TRPP2-CT-HIS to GST-LBD. We investigated further whether interaction was purely electrostatic or sequence-specific using a scrambled peptide of the acidic clus-

<sup>5</sup> B. Devogelaere, N. Nadif Kasri, E. Sammels, G. Bultynck, L. Missiaen, J. B. Parys, and H. De Smedt, unpublished results.

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**FIGURE 3. Identification of the sequences involved in the interaction between TRPP2-CT and IP<sub>3</sub>R-LBD.** A, GST pull-down using GST, GST-LBD, GST-LBD DDFD<sup>54</sup>, or GST-LBD AAFA<sup>54</sup>, and TRPP2-CT-HIS or TRPP2-NT-HIS. The upper panel shows Ponceau red staining of the input GST fusion proteins. The lower immunoblots show the staining for the HIS fusion proteins. B, GST pull-down using GST or GST-suppressor (aa 1–225) of IP<sub>3</sub>R3 or IP<sub>3</sub>R1 and full-length TRPP2 or TRPP2-CT-HIS. The upper panel shows Ponceau red staining of the input GST fusion proteins. The lower immunoblots show the staining for TRPP2-CT-HIS or full-length TRPP2. C, GST pull-down using GST-LBD and TRPP2-CT-HIS in the presence of acidic cluster peptide (AC) (10, 50, and 100  $\mu\text{M}$ ), the phosphomimetic S810D peptide (AC S<sup>810D</sup>) (50  $\mu\text{M}$ ), and the mutant peptide where four negatively charged residues are replaced by alanine (AC mutant) (50  $\mu\text{M}$ ). The upper immunoblot shows Ponceau red staining of the input proteins. The lower immunoblot shows the staining for TRPP2-CT-HIS. The quantification is averaged for three independent experiments and expressed in percent of the binding in the absence of peptides (mean  $\pm$  S.E.). D, GST pull-down using GST-LBD and TRPP2-CT-HIS in the presence of 100  $\mu\text{M}$  mutant acidic cluster peptide (AC mutant), scrambled acidic cluster peptide (AC Scr), and acidic cluster peptide itself (AC). The upper immunoblot shows Ponceau red staining of the input proteins. The lower immunoblot shows staining for TRPP2-CT-HIS. The quantification is averaged for three independent experiments and expressed in % of the binding in the absence of peptides (mean  $\pm$  S.E.).

ter (AC Scr) in GST pull-down assays (Fig. 3D). Addition of the scrambled acidic cluster peptide resulted in partial disturbance of the interaction between GST-LBD and TRPP2-CT, however, to a much lesser extent than the acidic cluster peptide. This finding demonstrates that the interaction is primarily based on electrostatic charges but that the exact location of these charges is also important and leads to a sequence-specific effect. Taken together, these data indicate that the acidic cluster in the C-terminal tail of TRPP2 and the positively charged cluster in the suppressor domain of the IP<sub>3</sub>R are crucial for interaction between the two proteins.

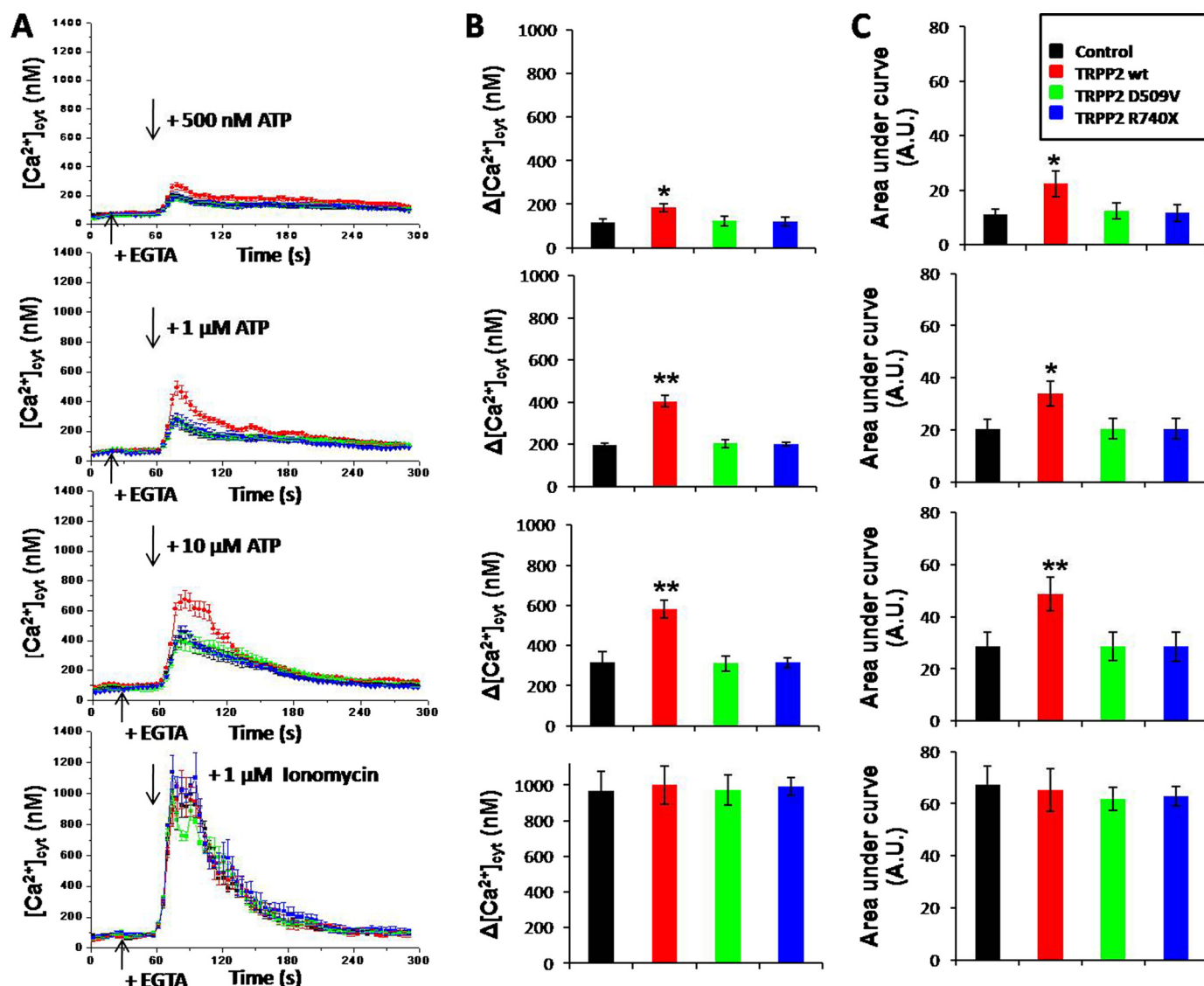
**TRPP2 Potentiates ATP-induced Intracellular  $\text{Ca}^{2+}$  Release in Intact Cells**—To study the effect of this molecular interaction on intracellular  $\text{Ca}^{2+}$  signaling, we used a TRPP2<sup>−/−</sup> renal epithelial cell line derived from TRPP2<sup>−/−</sup> mice (30) representing a zero-background system to study the effects mediated by wild-type TRPP2 or TRPP2 mutants. To re-introduce TRPP2 in these cells with high efficiency and an adjustable level of expression, we constructed an adenovirus encoding TRPP2. As shown on the Western blot and the confocal images (supplemental Fig. S3), the expression level of TRPP2 correlated with the virus titer. Adenoviral expressed TRPP2 was mainly localized in the ER. We chose a moderate TRPP2 expression, comparable with the endogenous levels of TRPP2 in renal epithelial cells (as shown in supplemental Fig. S4, lanes 1 and 4). This also excludes the need for making stable cell lines and the risk for concomitant selection of compensatory mutants. TRPP2<sup>−/−</sup> cells were transduced with a control virus or a TRPP2 virus to re-introduce wild-type

TRPP2, and cultured in 96-well plates. In intact cells, we examined the intracellular  $\text{Ca}^{2+}$  release upon agonist (ATP) addition by monitoring the  $[\text{Ca}^{2+}]_{\text{cyt}}$  in a microplate reader. Extracellular  $\text{Ca}^{2+}$  was buffered by addition of 3 mM EGTA at the beginning of each assay. For each ATP concentration (500 nM, 1  $\mu\text{M}$ , and 10  $\mu\text{M}$ ), a significant potentiation of the  $\text{Ca}^{2+}$  release was observed in cells expressing TRPP2 compared with cells treated with the control virus (Fig. 4A, black and red traces). Both the amplitude (Fig. 4B) and the area under the curve (Fig. 4C) were significantly increased for all three ATP concentrations used. At a maximal dose of ATP (100  $\mu\text{M}$ ) there was, however, no longer a difference in  $\text{Ca}^{2+}$  release between the cells expressing TRPP2 compared with the cells treated with control virus (supplemental Fig. S5). The maximal releasable  $\text{Ca}^{2+}$  induced by 1  $\mu\text{M}$  ionomycin was also not significantly altered (Fig. 4).

**TRPP2 Potentiates IICR in Permeabilized Cells**—The effect of TRPP2 on IICR was measured in plasma membrane-permeabilized cells loaded with the ER-resident  $\text{Ca}^{2+}$  indicator Mag-Fluo4. There was no significant difference in the loading of the ER with  $\text{Ca}^{2+}$  prior to the addition of IP<sub>3</sub> in cells treated with (i) a control virus, (ii) a TRPP2 virus, (iii) a TRPP2 D509V virus, and (iv) a TRPP2 R740X virus (discussed later) (supplemental Fig. S6). Therefore, the  $F/F_0$  data were normalized by setting the maximal  $[\text{Ca}^{2+}]_{\text{ER}}$  (as  $F/F_0$ ) prior to the IP<sub>3</sub> addition at 100%.

TG (10  $\mu\text{M}$ ) alone was added to investigate whether TRPP2 could mediate a passive leak of  $\text{Ca}^{2+}$  from the ER. We could not detect a significant difference in the rate of  $[\text{Ca}^{2+}]_{\text{ER}}$  decrease between control cells and cells expressing TRPP2 (Fig. 5A). Fur-

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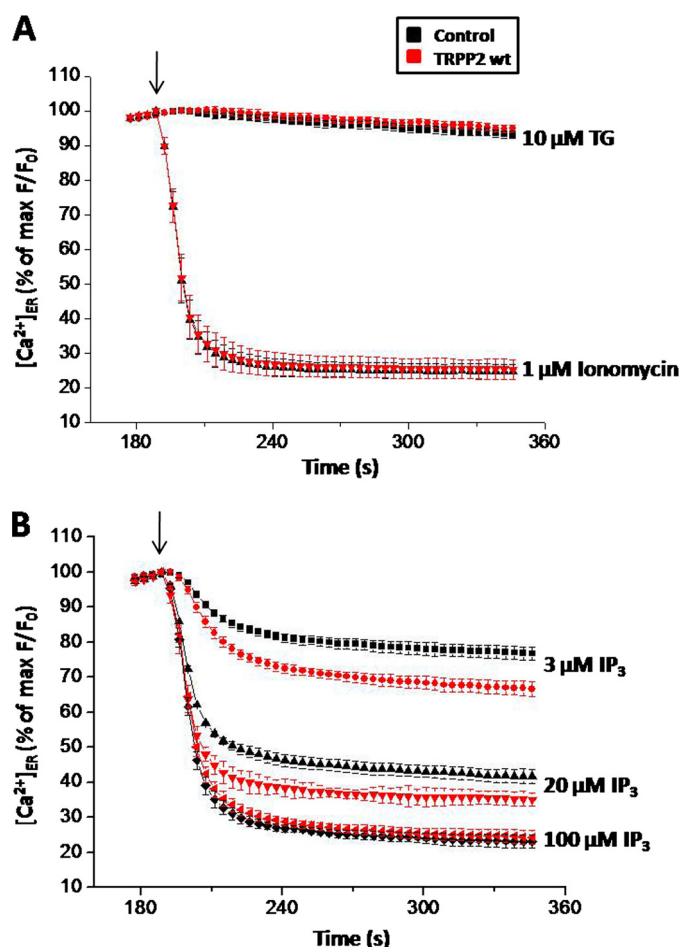
**FIGURE 4. Effect of TRPP2 and mutants on agonist-induced  $\text{Ca}^{2+}$  release in intact cells.** TRPP2<sup>-/-</sup> cells were treated with either control virus (black) (titer 1/1000) or a TRPP2 virus to re-introduce wild-type TRPP2 (red) (titer 1/1000) or mutants D509V (green) (titer 1/704) and R740X (blue) (titer 1/1124). The ratio of emitted fluorescence of Fura2 was monitored and the three different [ATP] or 1  $\mu\text{M}$  ionomycin were added as indicated in the presence of 3 mM EGTA.  $[\text{Ca}^{2+}]_{\text{cyt}}$  was derived after *in situ* calibration. Panel A shows the averaged traces of at least three independent experiments. Panel B shows the increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  (nM) after agonist addition, averaged for at least three independent experiments. Panel C shows the quantification of the area under the curve in arbitrary units (A.U.). Results are shown as mean  $\pm$  S.E. Statistically significant differences are labeled with: \*,  $p < 0.05$ , and \*\*,  $p < 0.01$ , using a Student's *t* test (paired two-tailed).

thermore, also in intact cells, we could not detect a significant difference in the rate of  $[\text{Ca}^{2+}]_{\text{cyt}}$  rise between control cells and cells expressing TRPP2 (supplemental Fig. S7) and the maximal ER  $\text{Ca}^{2+}$  content was not changed (supplemental Fig. S7, 100 s). The maximal releasable  $\text{Ca}^{2+}$  in permeabilized cells, determined by adding 1  $\mu\text{M}$  ionomycin, was the same for both conditions (Fig. 5A). However, TRPP2 potentiated IICR, because there was increased IICR in cells expressing TRPP2 compared with cells treated with the control virus (without TRPP2) when submaximal doses of  $\text{IP}_3$  were applied (Fig. 5B). Application of a saturating dose of  $\text{IP}_3$  (100  $\mu\text{M}$ ) no longer resulted in increased IICR in cells expressing TRPP2. A complete dose-response curve was fitted according to the Hill equation. The Hill coefficient and  $V_{\text{max}}$  remained unchanged, but the  $\text{EC}_{50}$  significantly changed

from  $5.77 \pm 0.39 \mu\text{M}$   $\text{IP}_3$  in control cells to  $3.09 \pm 0.05 \mu\text{M}$   $\text{IP}_3$  in cells expressing TRPP2 ( $p < 0.05$ ) (Table 1).

**Stimulation of IICR Is Dependent on TRPP2-channel Function and Interaction with the  $\text{IP}_3$ R**—To elucidate whether TRPP2 channel activity was involved in this effect on IICR, we made a D509V TRPP2 mutant. This pathological missense mutation of a single amino acid in the third membrane-spanning domain results in complete loss of TRPP2 channel activity (15). This mutant retained the ER localization and C-terminal-mediated protein interactions and regulatory domains. A second pathological R740X-truncated mutant, which lost the interaction site with the  $\text{IP}_3$ R, was made to verify whether the effect on IICR was dependent on the interaction between TRPP2 and  $\text{IP}_3$ R. This mutant still showed channel activity, although some channel properties like the regulation by  $\text{Ca}^{2+}$





**FIGURE 5. Effect of TRPP2 on  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release in permeabilized cells.** TRPP2 $^{-/-}$  cells were treated with either a control virus (black) (titer 1/1000) or a TRPP2 virus (red) to re-introduce wild-type TRPP2 (titer 1/1000). The fluorescence signal ( $F/F_0$ ) of the ER-resident  $\text{Ca}^{2+}$  indicator MagFluo4 was monitored. After permeabilizing the cells and loading the ER with  $\text{Ca}^{2+}$ ,  $\text{IP}_3$ , TG, or ionomycin were applied as indicated.  $\text{IP}_3$  was given in the presence of 10  $\mu\text{M}$  TG to prevent re-uptake of  $\text{Ca}^{2+}$  into the ER. *Panel A* shows the  $[\text{Ca}^{2+}]_{\text{ER}}$  (as % of max  $F/F_0$ ) after application of 10  $\mu\text{M}$  TG or 1  $\mu\text{M}$  ionomycin. *Panel B* shows the  $[\text{Ca}^{2+}]_{\text{ER}}$  (as % of max  $F/F_0$ ) after application of 3, 20, or 100  $\mu\text{M}$   $\text{IP}_3$ . Mean  $\pm$  S.E. of at least three independent experiments are shown.

**TABLE 1**

Fitting parameters according to the Hill equation of the  $\text{IP}_3$  dose-response curves

Using 8 different  $\text{IP}_3$  concentrations (250 and 500 nM, and 1, 3, 5, 20, 50, and 100  $\mu\text{M}$ ), a complete  $\text{IP}_3$  dose-response curve for cells expressing wild-type TRPP2 or control cells was fitted according to the Hill equation. The parameters  $\text{EC}_{50}$ , Hill coefficient, and  $V_{\text{max}}$  are depicted. Results are shown as mean  $\pm$  S.E. of three independent experiments.

	Control	TRPP2 wild-type	<i>p</i> value
$\text{EC}_{50}$	$5.77 \pm 0.39$	$3.09 \pm 0.05$	0.016 <sup>a</sup>
Hill coefficient	$1.08 \pm 0.09$	$1.15 \pm 0.15$	0.435
$V_{\text{max}}$	$105.73 \pm 5.11$	$103.22 \pm 4.33$	0.085

<sup>a</sup> Statistically significant differences with  $p < 0.05$  using a Student's *t* test (paired two-tailed).

are altered, possibly due to loss of the EF-hand (15, 39). In supplemental Fig. S4, the adenoviral expression and subcellular localization of wild-type TRPP2 and the two mutants are shown on a Western blot and in confocal images. In contrast to wild-type TRPP2, none of the mutants potentiated intracellular  $\text{Ca}^{2+}$  release in intact cells upon addition of 500 nM, 1  $\mu\text{M}$ , or 10  $\mu\text{M}$  ATP compared with control cells (Fig. 4). The maximal

releasable  $\text{Ca}^{2+}$  induced by 1  $\mu\text{M}$  ionomycin was not significantly different.

Also in permeabilized cells, IICR was no longer potentiated in cells expressing the mutants as compared with wild-type TRPP2 (Fig. 6A). The decrease of the  $[\text{Ca}^{2+}]_{\text{ER}}$  upon addition of 3  $\mu\text{M}$   $\text{IP}_3$  is shown. This value amounted to  $27.5 \pm 2.1\%$  in control cells,  $38.9 \pm 2.7\%$  in TRPP2-expressing cells,  $25.3 \pm 1.4\%$  in TRPP2 D509V-expressing cells, and  $26.0 \pm 1.2\%$  in TRPP2 R740X-expressing cells (Fig. 6A). The experiment with the channel-dead mutant indicates that the potentiation of IICR was dependent on TRPP2 channel activity. The results with the R740X mutant suggest also that the TRPP2- $\text{IP}_3\text{R}$  interaction was needed for this potentiation of IICR, although it cannot be completely excluded that the loss of potentiation of IICR was due to altered TRPP2 channel properties. Therefore a second approach was used to validate the role of the interaction between TRPP2 and the  $\text{IP}_3\text{R}$  in intracellular  $\text{Ca}^{2+}$  signaling (Fig. 6B). Disruption of the interaction between TRPP2 and  $\text{IP}_3\text{R}$  by addition of the AC peptide (*cf.* Fig. 3C) prevented potentiation of IICR in TRPP2-expressing cells in response to 1  $\mu\text{M}$   $\text{IP}_3$ , whereas this potentiation was not prevented by AC mutant peptide (Fig. 6B). These peptides had no effect on IICR in control cells. These results confirm that an interaction between TRPP2 and  $\text{IP}_3\text{R}$  was required to potentiate intracellular  $\text{Ca}^{2+}$  release upon  $\text{IP}_3\text{R}$  activation.

We hypothesize that TRPP2 can be activated by an initial IICR via a  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release (CICR) mechanism. To substantiate this hypothesis, we used BAPTA, a faster  $\text{Ca}^{2+}$  buffer than EGTA, at a high concentration (20 mM BAPTA instead of 1 mM EGTA) in the  $\text{Ca}^{2+}$  assay in permeabilized cells. Under these conditions, TRPP2 no longer potentiated the IICR after addition of 5  $\mu\text{M}$   $\text{IP}_3$  (Fig. 6C). It is noted that the extent of the release was lower when using BAPTA buffering, compared with EGTA buffering. The reason for this is that BAPTA can slightly inhibit IICR (40). To further rule out that TRPP2 did not simply function as an additional CICR mechanism but required an initial IICR, we applied submaximal doses of ionomycin in intact and permeabilized cells. This provoked a general rise in  $[\text{Ca}^{2+}]_{\text{cyt}}$  and a decrease in  $[\text{Ca}^{2+}]_{\text{ER}}$ , but there was no potentiation of the  $\text{Ca}^{2+}$  release in cells expressing TRPP2, compared with cells treated with the control virus (Fig. 7). We therefore conclude that a local  $\text{Ca}^{2+}$  release as produced by  $\text{IP}_3$  is required for inducing CICR via TRPP2, whereas a global  $\text{Ca}^{2+}$  increase as by ionomycin cannot produce this CICR.

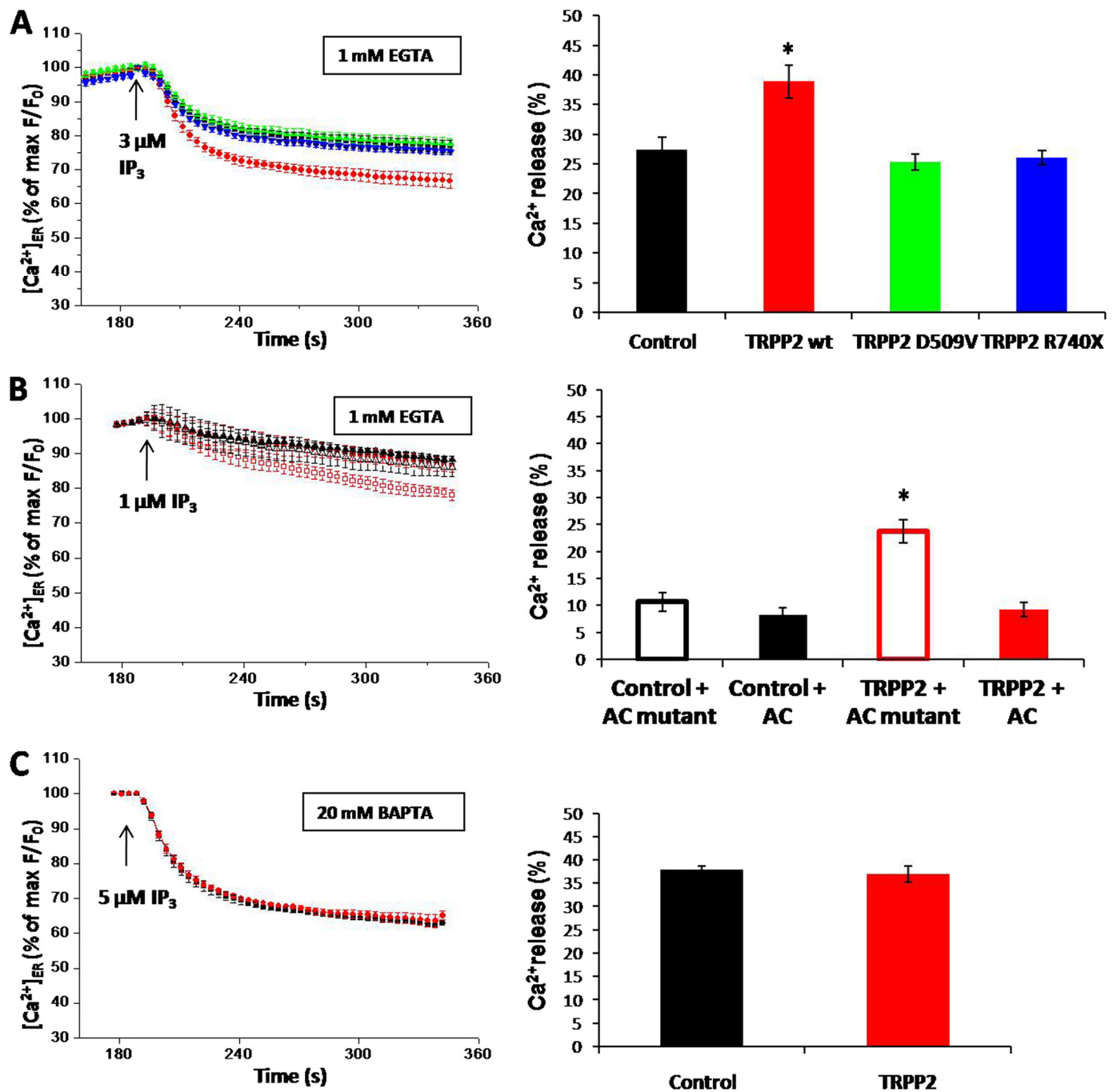
Taken together, these data suggest that TRPP2 and the  $\text{IP}_3\text{R}$  form a signaling complex at the ER, thereby fine-tuning and coordinating  $\text{Ca}^{2+}$  signaling in a microdomain of intracellular  $\text{Ca}^{2+}$  release. The close proximity of TRPP2 channels and  $\text{IP}_3\text{Rs}$  in a protein complex thereby allows the amplification of  $\text{IP}_3\text{R}$  originating  $\text{Ca}^{2+}$  signals via a local CICR mechanism.

## DISCUSSION

The main finding of this study was that endogenous TRPP2- $\text{IP}_3\text{R}$  signaling complexes are responsible for the TRPP2-dependent potentiation of IICR. The novel findings in this work are: (i) the identification of residues responsible for the interaction, on TRPP2 as well as on the  $\text{IP}_3\text{R}$ , which can now be targeted to disrupt or enhance the interaction; (ii) the mechanism



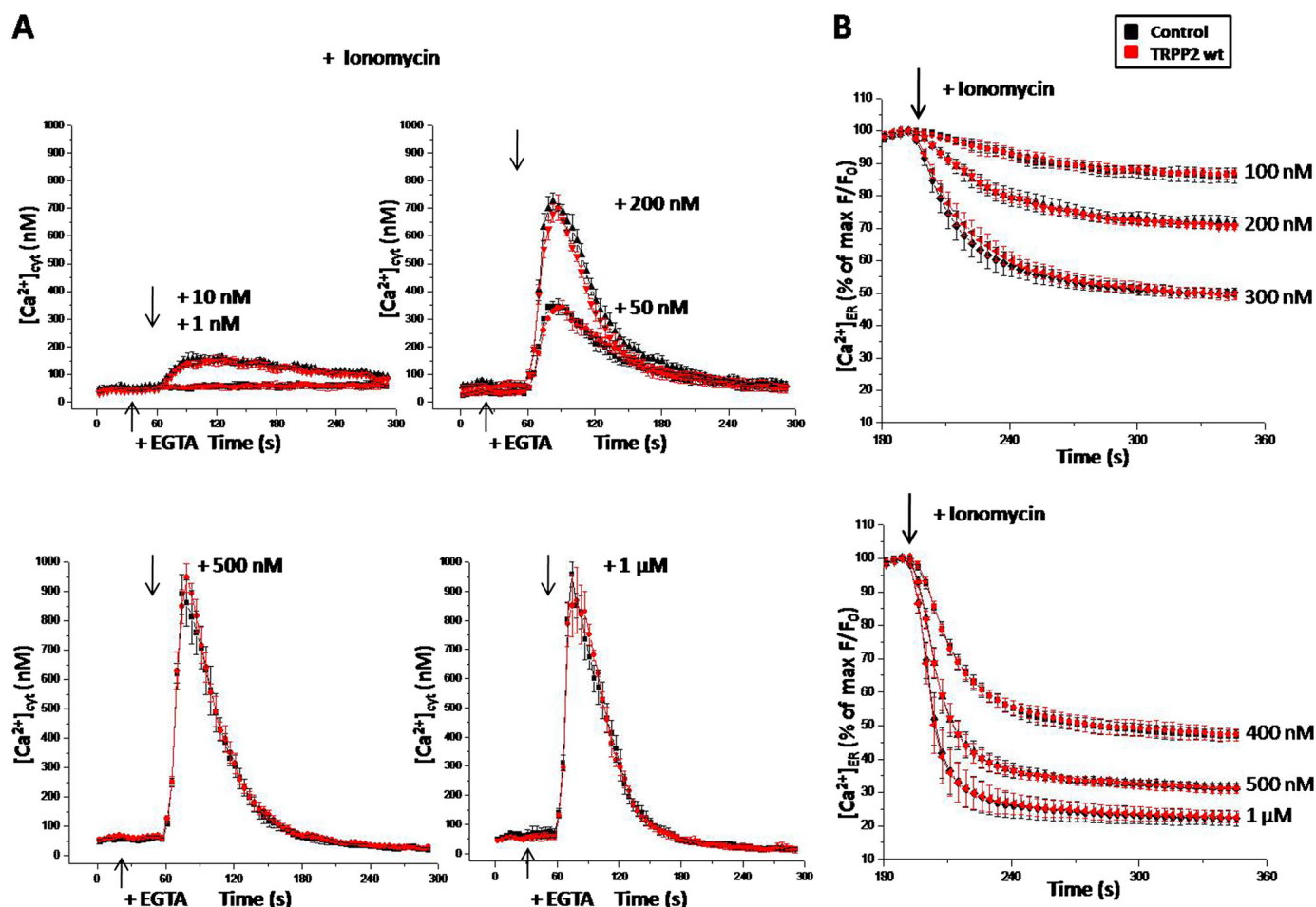
## Polycystin-2 and Intracellular $\text{Ca}^{2+}$ Signaling



**FIGURE 6. Effect of TRPP2 mutants, competing peptides, and altered  $\text{Ca}^{2+}$  buffering conditions on  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  release in permeabilized cells.** A, TRPP2 $^{-/-}$  cells were treated with either a control virus (black) (titer 1/1000) or a TRPP2 virus to re-introduce wild-type TRPP2 (red) (titer 1/1000) or mutants D509V (green) (titer 1/704) and R740X (blue) (titer 1/1124). Experiments were performed as described in the legend to Fig. 5 with 3  $\mu\text{M}$   $\text{IP}_3$  and with 1 mM EGTA for  $\text{Ca}^{2+}$  buffering. B, TRPP2 $^{-/-}$  cells were treated with either a control virus (black) (titer 1/1000) or a TRPP2 virus (red) to re-introduce wild-type TRPP2 (titer 1/1000) and assayed as described in the legend to Fig. 5. 1  $\mu\text{M}$   $\text{IP}_3$  was added to the cells in the presence of 10  $\mu\text{M}$  acidic cluster peptide (AC, closed symbols) or a mutant peptide (AC mutant, open symbols). Experiment was performed in the presence of 1 mM EGTA for  $\text{Ca}^{2+}$  buffering. C, TRPP2 $^{-/-}$  cells were treated with either a control virus (black) (titer 1/1000) or a TRPP2 virus to re-introduce wild-type TRPP2 (red) (titer 1/1000). Experiments were performed as described in the legend to Fig. 5 with 5  $\mu\text{M}$   $\text{IP}_3$ , except that the  $\text{Ca}^{2+}$  was buffered with 20 mM BAPTA instead of 1 mM EGTA. The left panels show the results as mean  $[\text{Ca}^{2+}]_{\text{ER}}$  (in % of max  $F/F_0$ ) for at least three independent experiments. The right panels show the percentage  $\text{Ca}^{2+}$  release (mean  $\pm$  S.E.). Data values were normalized to the ionomycin-induced  $\text{Ca}^{2+}$  release, which was taken as 100%, after subtracting the TG-induced  $\text{Ca}^{2+}$  release. Statistically significant differences are labeled with: \*,  $p < 0.05$ , using a Student's  $t$  test (paired two-tailed).

responsible for the potentiated  $\text{Ca}^{2+}$  release, which was due to TRPP2 activity rather than to altered  $\text{IP}_3\text{R}$  activity and that required a close association between TRPP2 and the  $\text{IP}_3\text{R}$  to allow for a  $\text{Ca}^{2+}$  microdomain responsible for the activation of TRPP2; (iii) the finding that TRPP2 was not responsible for a

passive  $\text{Ca}^{2+}$  leak from the ER; and (iv) the demonstration of CICR via TRPP2 in a cellular model. We conclude that an acidic cluster (aa 810–818) in the cytosolic C-terminal tail of TRPP2 and a positively charged cluster (aa 51–54) in the suppressor region of the LBD in the N terminus of the  $\text{IP}_3\text{R}$  were required



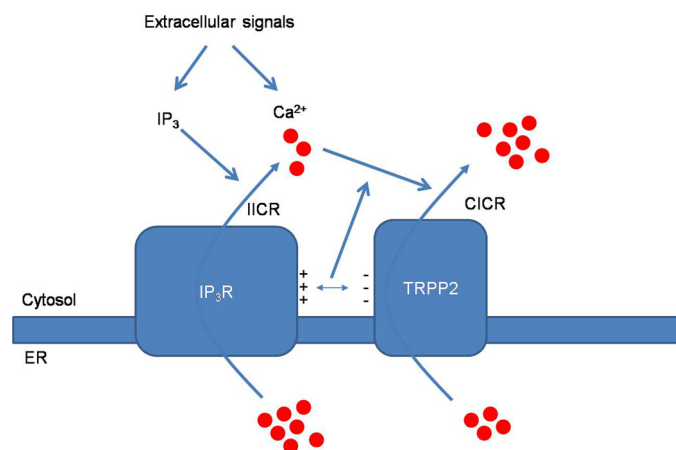
**FIGURE 7. Effect of TRPP2 on ionomycin-induced  $\text{Ca}^{2+}$  release in intact and permeabilized cells.** TRPP2<sup>-/-</sup> cells were treated with either control virus (black) (titer 1/1000) or a TRPP2 virus to re-introduce wild-type TRPP2 (red) (titer 1/1000). *A*, the ratio of emitted fluorescence of Fura2 was monitored and six different ionomycin concentrations were added as indicated in the presence of 3 mM EGTA.  $[\text{Ca}^{2+}]_{\text{cyt}}$  was derived after *in situ* calibration. *Panel A* shows the averaged traces of at least three independent experiments. *B*, experiments were performed as described in the legend to Fig. 5 with the indicated ionomycin concentrations and with 1 mM EGTA for  $\text{Ca}^{2+}$  buffering. Results are shown as mean  $[\text{Ca}^{2+}]_{\text{ER}}$  (as % of max  $F/F_0$ )  $\pm$  S.E. for three independent experiments.

for their interaction. As a functional consequence of this interaction, we found that TRPP2 potentiated IICR. In a previous study (23) it was postulated that the C-terminal part of TRPP2 could alter the activity of the  $\text{IP}_3\text{R}$ . This conclusion was based on overexpression of the truncated C terminus or the channel-dead mutant in oocytes. Our data, however, do not provide evidence for a changed activity of the  $\text{IP}_3\text{R}$  as the channel-dead mutant of TRPP2 had no effect on IICR in our experimental model, where we have carefully avoided overexpression to physiologically irrelevant levels. Rather, the enhanced  $\text{Ca}^{2+}$  release was most likely due to activation of CICR via TRPP2. This was concluded from our observation that the potentiation of IICR by TRPP2 was abolished in strong  $\text{Ca}^{2+}$ -buffering conditions using 20 mM BAPTA, which reduces the microdomain of the elevated free  $[\text{Ca}^{2+}]$  (41). The difference between slow (EGTA) or fast (BAPTA)  $\text{Ca}^{2+}$  buffering is an established method to estimate the importance of the local  $[\text{Ca}^{2+}]_{\text{cyt}}$  rise in a microdomain close to  $\text{Ca}^{2+}$ -release channels. For this observed CICR, activation by a local  $[\text{Ca}^{2+}]_{\text{cyt}}$  rise following IICR and the close proximity of the  $\text{IP}_3\text{R}$  via a direct interaction with TRPP2 appeared to be required. The latter was also evident from our observation that a truncated TRPP2, which lacks the  $\text{IP}_3\text{R}$ -binding site, did not provoke

this potentiation of IICR. Moreover, the effect was also abolished by a peptide competing for the  $\text{IP}_3\text{R}$ -binding site. An additional support for the importance of a close interaction between TRPP2 and the  $\text{IP}_3\text{R}$  stems from the observation that a global  $[\text{Ca}^{2+}]_{\text{cyt}}$  rise as provoked by ionomycin or TG did not result in an increased TRPP2-induced  $\text{Ca}^{2+}$  signal. We propose a model (Fig. 8) where upon cell stimulation  $\text{IP}_3$  is produced and activates the  $\text{IP}_3\text{R}$ , which leads to a local  $[\text{Ca}^{2+}]_{\text{cyt}}$  rise that subsequently can activate the TRPP2 channel as a CICR channel. Previous lipid-bilayer experiments have demonstrated regulation of the open probability of the TRPP2 channel by  $\text{Ca}^{2+}$  (25).

Besides important functions of TRPP2 at the plasma membrane and the primary cilia, we now suggest another critical function for TRPP2 in the ER. We propose the existence of a signaling microdomain that requires a functional TRPP2/ $\text{IP}_3\text{R}$ -protein complex to facilitate CICR by TRPP2. TRPP2 participates in an intracellular channel complex and potentiates intracellular  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  signaling. It has been shown in lipid bilayer experiments that TRPP2 is a  $\text{Ca}^{2+}$ -dependent cation channel, whose activity shows a bell-shaped dependence on  $[\text{Ca}^{2+}]_{\text{cyt}}$  (15, 25). The activation phase of the CICR via TRPP2 was reported to have micromolar affinity (25). Given the rela-

## Polycystin-2 and Intracellular $\text{Ca}^{2+}$ Signaling



**FIGURE 8. Proposed model.** Upon cell stimulation,  $\text{IP}_3$  is produced and activates the  $\text{IP}_3\text{R}$  leading to a local  $[\text{Ca}^{2+}]_{\text{cyt}}$  rise that subsequently activates the TRPP2 channel as a CICR channel. We suggest that a signaling microdomain, where TRPP2 interacts with the  $\text{IP}_3\text{R}$  through the charged residues, is required to facilitate CICR by TRPP2.

tively high  $[\text{Ca}^{2+}]$  predicted at the mouth of the  $\text{IP}_3\text{R}$  at the ER surface (34, 42), a micromolar affinity for  $\text{Ca}^{2+}$  may allow TRPP2 to sense such local changes in  $[\text{Ca}^{2+}]_{\text{cyt}}$ . Recent studies on the structure of the EF-hand within the cytosolic C terminus of TRPP2 have brought new insights into the conformational changes that occur upon  $\text{Ca}^{2+}$  binding (6, 8). However, the direct link between  $\text{Ca}^{2+}$  binding to the EF-hand of TRPP2 and its  $\text{Ca}^{2+}$ -dependent channel regulation has not been demonstrated yet and requires further research. Furthermore, loss of phosphorylation of TRPP2 (Ser<sup>810</sup>) can shift the bell-shaped  $[\text{Ca}^{2+}]_{\text{cyt}}$  dependence of TRPP2 channel activity to the right (25). Given our results from the binding assay with the phosphomimetic acidic cluster peptide (Fig. 3B), reduced binding of TRPP2 to the  $\text{IP}_3\text{R}$  may be one of the mechanisms responsible for the observed decrease in sensitivity to  $\text{Ca}^{2+}$  upon dephosphorylation.

Intracellular  $\text{Ca}^{2+}$  signaling modulated by TRPP2 is important for regulating various cellular processes including cell proliferation. Loss of TRPP2 results in increased cell proliferation in ADPKD and this is one of the mechanisms leading to cyst formation (5, 43). It has been reported that ADPKD cystic cells have a lower  $[\text{Ca}^{2+}]_{\text{cyt}}$ , probably due to a loss of TRPP2 channel function (44). Increased levels of cAMP and cAMP-dependent genes (such as aquaporin 2) are also a common finding in the kidneys of different ADPKD animal models. In ADPKD or under  $\text{Ca}^{2+}$  deprivation conditions, cAMP stimulates cell proliferation in an Src-, Ras-, and B-Raf-dependent manner (45). In view of the role of  $\text{Ca}^{2+}$  in the regulation of cAMP metabolism via  $\text{Ca}^{2+}$ -inhibitable adenylyl cyclase 6 and/or via  $\text{Ca}^{2+}$ -dependent phosphodiesterase-1, it has already been suggested that alterations in intracellular  $\text{Ca}^{2+}$  homeostasis account for the increase in cAMP levels (5). It is tempting to speculate that the increased  $\text{Ca}^{2+}$  signaling in  $\text{IP}_3\text{R}$ /TRPP2 microdomains may be operative in controlling cellular cAMP levels by  $\text{Ca}^{2+}$  inhibitable adenylyl cyclases. Taken together, the previous findings strongly suggest that the mechanisms regulating  $[\text{Ca}^{2+}]_{\text{cyt}}$  form potential therapeutic targets for ADPKD.

Not only proliferation, but also apoptosis is an important  $\text{Ca}^{2+}$ -regulated cellular process that is impaired in ADPKD,

although this might be rather a consequence than a cause of cyst formation. Recently, Wegierski *et al.* (27) have proposed a role for ER-located TRPP2 in apoptosis. They have found that TRPP2 can lower the ER  $\text{Ca}^{2+}$  content, thereby protecting against apoptosis. In contrast to our data and previous work (15, 23), they observed that TRPP2 diminished cytosolic and mitochondrial  $\text{Ca}^{2+}$  signals induced by  $\text{IP}_3\text{R}$  activation. Because they observed a decreased ER  $\text{Ca}^{2+}$  content in TRPP2 overexpressing cells, it appeared that those cells have a diminished  $\text{Ca}^{2+}$  release upon  $\text{IP}_3\text{R}$  activation. In contrast, we could neither detect a change in  $[\text{Ca}^{2+}]_{\text{ER}}$  nor in passive  $\text{Ca}^{2+}$  leak (Fig. 5A and supplemental Fig. S7), and we consistently observed an increase in IICR (Fig. 5B). A difference in our experimental cell model is that we did not overexpress TRPP2, but chose expression levels comparable with endogenous levels of TRPP2 (supplemental Fig. S4). In basal conditions, the TRPP2 channel is expected to be silent and inhibited by the endogenous protein syntaxin-5 (46). When TRPP2 is overexpressed, it is possible that the endogenous level of syntaxin-5 is not sufficient to maintain this basal inhibition of TRPP2.

In summary, we observed a clear interaction between TRPP2 and the  $\text{IP}_3\text{R}$  and identified a conserved positively charged cluster in the N-terminal suppressor domain of the  $\text{IP}_3\text{R}$  and an acidic cluster located at the end of the ER-retention signal in the C terminus of TRPP2 as being crucial for their interaction. When full-length TRPP2 was re-introduced in TRPP2<sup>-/-</sup> mouse renal epithelial cells, there was a clear potentiation of agonist-induced intracellular  $\text{Ca}^{2+}$  release in intact cells and IICR in permeabilized cells. Further analysis using pathological mutants of TRPP2 and competing peptides revealed that this effect on IICR was dependent on the TRPP2 channel function and on the interaction with the  $\text{IP}_3\text{R}$ . We propose a model in which the TRPP2 channel itself is activated as a CICR channel by a local  $[\text{Ca}^{2+}]_{\text{cyt}}$  rise generated by IICR (Fig. 8).

We conclude that a signaling complex involving TRPP2 and the  $\text{IP}_3\text{R}$  is important for modulating intracellular  $\text{Ca}^{2+}$  signaling. Disturbance of this interaction, which occurs in pathologically relevant mutants of TRPP2, will lead to altered intracellular  $\text{Ca}^{2+}$  homeostasis and might contribute to the development of ADPKD caused by loss-of-function mutations in TRPP2.

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## REFERENCES

- Gabow, P. A., and Grantham, J. J. (1997) in *Diseases of the Kidney* (Schrier, R. W., and Gottschalk, C. W., eds) 6th Ed., pp 521–560, Little Brown and Company, Boston, MA
- Mochizuki, T., Wu, G., Hayashi, T., Xenophontos, S. L., Veldhuisen, B., Saris, J. J., Reynolds, D. M., Cai, Y., Gabow, P. A., Pierides, A., Kimberling, W. J., Breuning, M. H., Deltas, C. C., Peters, D. J., and Somlo, S. (1996)



- Science* **272**, 1339–1342
3. Harris, P. C. (2009) *J. Am. Soc. Nephrol.* **20**, 1188–1198
4. Wu, G., and Somlo, S. (2000) *Mol. Genet. Metab.* **69**, 1–15
5. Torres, V. E., and Harris, P. C. (2009) *Kidney Int.* **76**, 149–168
6. Schumann, F., Hoffmeister, H., Bader, R., Schmidt, M., Witzgall, R., and Kalbitzer, H. R. (2009) *J. Biol. Chem.* **284**, 24372–24383
7. Cai, Y., Maeda, Y., Cedzich, A., Torres, V. E., Wu, G., Hayashi, T., Mochizuki, T., Park, J. H., Witzgall, R., and Somlo, S. (1999) *J. Biol. Chem.* **274**, 28557–28565
8. Celić, A., Petri, E. T., Demeler, B., Ehrlich, B. E., and Boggon, T. J. (2008) *J. Biol. Chem.* **283**, 28305–28312
9. Yu, Y., Ulbrich, M. H., Li, M. H., Buraei, Z., Chen, X. Z., Ong, A. C., Tong, L., Isacoff, E. Y., and Yang, J. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106**, 11558–11563
10. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A. K., Piontek, K., Tsiokas, L., Sukhatme, V. P., Guggino, W. B., and Germino, G. G. (2000) *Nature* **408**, 990–994
11. Nauli, S. M., Alenghat, F. J., Luo, Y., Williams, E., Vassilev, P., Li, X., Elia, A. E., Lu, W., Brown, E. M., Quinn, S. J., Ingber, D. E., and Zhou, J. (2003) *Nat. Genet.* **33**, 129–137
12. Tsiokas, L., Arnould, T., Zhu, C., Kim, E., Walz, G., and Sukhatme, V. P. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 3934–3939
13. Bai, C. X., Giamarchi, A., Rodat-Despoix, L., Padilla, F., Downs, T., Tsiokas, L., and Delmas, P. (2008) *EMBO Rep.* **9**, 472–479
14. Köttgen, M., Buchholz, B., Garcia-Gonzalez, M. A., Kotsis, F., Fu, X., Doerken, M., Boehlke, C., Steffl, D., Tauber, R., Wegierski, T., Nitschke, R., Suzuki, M., Kramer-Zucker, A., Germino, G. G., Watnick, T., Prenen, J., Nilius, B., Kuehn, E. W., and Walz, G. (2008) *J. Cell Biol.* **182**, 437–447
15. Koulen, P., Cai, Y., Geng, L., Maeda, Y., Nishimura, S., Witzgall, R., Ehrlich, B. E., and Somlo, S. (2002) *Nat. Cell Biol.* **4**, 191–197
16. Tsiokas, L., Kim, S., and Ong, E. C. (2007) *Cell Signal.* **19**, 444–453
17. Giamarchi, A., Padilla, F., Coste, B., Raoux, M., Crest, M., Honoré, E., and Delmas, P. (2006) *EMBO Rep.* **7**, 787–793
18. Köttgen, M. (2007) *Biochim. Biophys. Acta* **1772**, 836–850
19. Geng, L., Okuhara, D., Yu, Z., Tian, X., Cai, Y., Shibasaki, S., and Somlo, S. (2006) *J. Cell Sci.* **119**, 1383–1395
20. Köttgen, M., Benzing, T., Simmen, T., Tauber, R., Buchholz, B., Felician-geli, S., Huber, T. B., Schermer, B., Kramer-Zucker, A., Höpker, K., Simmen, K. C., Tschucke, C. C., Sandford, R., Kim, E., Thomas, G., and Walz, G. (2005) *EMBO J.* **24**, 705–716
21. Köttgen, M., and Walz, G. (2005) *Pflugers Arch.* **451**, 286–293
22. Anyatonwu, G. I., Estrada, M., Tian, X., Somlo, S., and Ehrlich, B. E. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104**, 6454–6459
23. Li, Y., Wright, J. M., Qian, F., Germino, G. G., and Guggino, W. B. (2005) *J. Biol. Chem.* **280**, 41298–41306
24. Li, Y., Santoso, N. G., Yu, S., Woodward, O. M., Qian, F., and Guggino, W. B. (2009) *J. Biol. Chem.* **284**, 36431–36441
25. Cai, Y., Anyatonwu, G., Okuhara, D., Lee, K. B., Yu, Z., Onoe, T., Mei, C. L., Qian, Q., Geng, L., Witzgall, R., Ehrlich, B. E., and Somlo, S. (2004) *J. Biol. Chem.* **279**, 19987–19995
26. Miyagi, K., Kiyonaka, S., Yamada, K., Miki, T., Mori, E., Kato, K., Numata, T., Sawaguchi, Y., Numaga, T., Kimura, T., Kanai, Y., Kawano, M., Wakamori, M., Nomura, H., Koni, I., Yamagishi, M., and Mori, Y. (2009) *J. Biol. Chem.* **284**, 34400–34412
27. Wegierski, T., Steffl, D., Kopp, C., Tauber, R., Buchholz, B., Nitschke, R., Kuehn, E. W., Walz, G., and Köttgen, M. (2009) *EMBO J.* **28**, 490–499
28. Lee, B., Vermassen, E., Yoon, S. Y., Vanderheyden, V., Ito, J., Alfandari, D., De Smedt, H., Parys, J. B., and Fissore, R. A. (2006) *Development* **133**, 4355–4365
29. Sienaert, I., Missiaen, L., De Smedt, H., Parys, J. B., Sipma, H., and Casteels, R. (1997) *J. Biol. Chem.* **272**, 25899–25906
30. Wu, G., D'Agati, V., Cai, Y., Markowitz, G., Park, J. H., Reynolds, D. M., Maeda, Y., Le, T. C., Hou, H., Kucherlapati, R., Edelmann, W., and Somlo, S. (1998) *Cell* **93**, 177–188
31. Grimm, D. H., Cai, Y., Chauvet, V., Rajendran, V., Zeltner, R., Geng, L., Avner, E. D., Sweeney, W., Somlo, S., and Caplan, M. J. (2003) *J. Biol. Chem.* **278**, 36786–36793
32. Parys, J. B., De Smedt, H., Missiaen, L., Bootman, M. D., Sienaert, I., and Casteels, R. (1995) *Cell Calcium* **17**, 239–249
33. Laude, A. J., Tovey, S. C., Dedos, S. G., Potter, B. V., Lummis, S. C., and Taylor, C. W. (2005) *Cell Calcium* **38**, 45–51
34. Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007) *Physiol. Rev.* **87**, 593–658
35. Devogelaere, B., Verbert, L., Parys, J. B., Missiaen, L., and De Smedt, H. (2008) *Cell Calcium* **43**, 17–27
36. Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1999) *J. Biol. Chem.* **274**, 316–327
37. Kasri, N. N., Bultynck, G., Smyth, J., Szlufcik, K., Parys, J. B., Callewaert, G., Missiaen, L., Fissore, R. A., Mikoshiba, K., and De Smedt, H. (2004) *Mol. Pharmacol.* **66**, 276–284
38. Li, X., Luo, Y., Starremans, P. G., McNamara, C. A., Pei, Y., and Zhou, J. (2005) *Nat. Cell Biol.* **7**, 1202–1212
39. Chen, X. Z., Segal, Y., Basora, N., Guo, L., Peng, J. B., Babakhanlou, H., Vassilev, P. M., Brown, E. M., Hediger, M. A., and Zhou, J. (2001) *Biochem. Biophys. Res. Commun.* **282**, 1251–1256
40. Richardson, A., and Taylor, C. W. (1993) *J. Biol. Chem.* **268**, 11528–11533
41. Hay, J. C. (2007) *EMBO Rep.* **8**, 236–240
42. Neher, E. (1998) *Neuron* **20**, 389–399
43. Chang, M.-Y., and Ong, A. C. (2008) *Nephron Physiol.* **108**, 1–7
44. Yamaguchi, T., Hempson, S. J., Reif, G. A., Hedge, A. M., and Wallace, D. P. (2006) *J. Am. Soc. Nephrol.* **17**, 178–187
45. Yamaguchi, T., Nagao, S., Wallace, D. P., Belibi, F. A., Cowley, B. D., Pelling, J. C., and Grantham, J. J. (2003) *Kidney Int.* **63**, 1983–1994
46. Geng, L., Boehmerle, W., Maeda, Y., Okuhara, D. Y., Tian, X., Yu, Z., Choe, C. U., Anyatonwu, G. I., Ehrlich, B. E., and Somlo, S. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 15920–15925